Experimental Biochemistry
Prof. Pritam Roy
Department of Chemistry
Indian Institute of Technology, Kharagpur

Lecture – 37
Enzyme Kinetics
(By using enzyme from apple juice) (Contd.)

(Refer Slide Time: 00:16)

So, this is the apple extract we have taken it in a falcon tube. Here we can see the deep brown color, ok. So, we are not going to use this entire solution for analysis, we going to dilute it. Basically, we are going to make it half dilute, ok; for that what we are going to make is that we are going to take another clean falcon, and we are going to pour around 20 ml of a 20 to 25 ml of this apple extract and the remaining we are going to dilute it with double distilled water. So, this color appears much more soothing as compared to the previous one. So, this craw colored craw yellow colored or rather bit brownish yellow colored solution, we are going to use for our analysis.

(Refer Slide Time: 01:39)

Now, let us proceed to the enzyme kinetics study. So, now let us start with the kinetics experiment for that we will be using a UV spectrophotometer. In the previous experimental (Refer Time: 01:46) course we have actually seen how to use this UV spectrophotometer, what are its function, about its compartment and etcetera all those things we have actually have seen there. Well, we have prepared the solution starting from catechol and inhibitors along with the enzyme extract should apple extract and now we will shift the experimental part. As you can see here this one is actually the UV instrument and the UV spectrophotometer out here is similar to that we are used earlier, but this is the defined type of instrument out here.

Now, the compartment and above the instrumental parameters and in how to setup this instrument how to use this instrument we have seen in the previous experimental classes; pretty same there in the software also. Now, what we can see out here in the software tool? This is the screen out here. We can see that this access is absorbance one and this one is the nanometer wavelength part.

Now, for kinetics study what we need to do is that we need to shift this one from the absorbance versus wavelength to absorbance versus time. For that how we can do is that we should go to window and fill it, from here we can see kinetics, photometric and spectrum, ok. From here we can actually select this kinetics. Once you select this kinetics we can see there is a change in this appearance of the software in the software screen out

here and here we can see this is absorbance part and here in the time and time is given seconds. Before beginning with this experiment we need to connect this one as we have seen in the previous UV spectrophotometer also. If you please connect out here, the other all those other parameters will get enabled.

Now, one important thing is there we need to get the parameters ready. Now, for this parameter we need to click this one M, this is similar to the previous UV spectrophotometer which we have used, this method 1 M and here it is the kinetic mode is fine the kinetic method. Various options where there like measurements sample preparation file etcetera. Let us see one by one what we get here. Now, here we can see in the timing mode one is given in auto and the other is given manual. So, in manual part what we can see is that the units basically we can change from second minute to hour. However, for doing kinetics experiment in biochemistry we have to generally stick to the second part; the next thing of a total time.

However, the total time is taking 300. Why? Because the cycle time and the number of readings. So, are basically if you take into account this two things you can land up to total time 300 seconds and these are the activity region 20 to 40.

However, we are not going into manual part will stick to auto part and the total time out here will be 5 minutes which is basically 300 seconds. So, here the units are second and total time is 300 and this we are not bothering with a cycle time and number of readings out here and the activity region is 20 and 40. This is 20 second to 40 second. These are by default, but; however, we you can change once you start with the experiment will show you later on.

Next thing we need to see here the wavelength; the wavelength given in nanometer. The number of wavelengths we need to study here either single or multiple here WL 1 minus WL 2 and WL 1 by WL 2 other modes are also shown. However, we are interested with single wavelength and the wavelength we are going to study is not 290, but 514 because we are going to see the effect on catechol using catechol enzyme that is the enzyme which catechol oxides enzyme which actually oxidizes catechol.

And, all other parameters we need not need to worry about, the other is the sample preparation and these are things which we do not require out here; file options which file we should save. So, it is pretty simple and it is same as that the previous one in the

spectrum file. We can select here; we are here in this NPTEL folder as you can see and we create enzyme kinetics course and we are giving the filename as KIN or basically kinetics these are for yourself convince and we are clicking OK.

Now, here one thing we can see these are the absorbance we are showing and we can change here from 0 and this one we can change to 1 and the time here we can see it is staring from 0 and it is going up to 300 seconds. Now, before beginning with this experiment what we can say is that we need cuvette. So, these are basically cuvettes.

(Refer Slide Time: 07:00)

The cuvettes are a quite familiar, we have seen in the previous biochemistry experimental classes where the cuvette here one side is actually clean and the other side is actually not clear and it is not transparent, and this cuvette we are going to use. This is actually 600 microlitre cuvette.

So, initially what we need to do? We need to get phosphate buffer filled in this cuvette before beginning with our experiment. Now, we will be using the phosphate buffer. The phosphate buffer will be using is pH 7.4 10 millimolar phosphate buffer. So, we had taken this micro pipette out here and this is the cuvette, ok. Now, I am taking out phosphate buffer that is 600 micro litre from here and gradually adding this in the cuvette. This whole procedure of how to add the solution in the cuvette we have seen in the previous classes I am not going into details out here.

(Refer Slide Time: 08:22)

Now, in the UV compartment you know there were two chambers; one is the control chamber, the other is the sample chamber. So, this one what we are going to do is that we are going to take a clean tissue paper and we are going to rub this clear side out here properly and open this lid and transfer it into the control compartment.

In a similar fashion, you are going to take 600 microlitre of phosphate buffer, put here. Clean this two sides properly and then transfer it into the other compartment that is the sample compartment, the sample holder basically, ok. So, the inner part of this UV compartment is pretty same to that of previous instrument. There are two holders one is for control and other is for sample.

Now, closing this thing and moving on to the screen, ok. Now, we have added this two buffer into the cuvette and placed it in the compartment. Now, we will go do this baseline correction so that we have taken the phosphate buffer in the cuvette and placed it in the compartment UV compartment out here we will do this baseline. And a baseline here is showing the start is actually 550 and the end is 530 and because it is scanning in the two regions that is 550, 530 and; however, our point of interest is actually 540. So, now, for that let us do the baseline correction, ok. So, we have set this our wavelength out here at 540. The scan will be taking place at this wavelength only.

Now, the second thing we need required is that the addition of subscript and the protein. So, this is the bit a crucial step and let us see how to carry out this process. Now, we will

adjust the concentration of subscript and enzyme within this mixture. In the beginning we have to add the subscript that is our catechol. So, the concentration of catechol we are using out here is actually 1 milli molar and for that we require around 30 microlitre of catechol. And the protein we are adding or rather the enzyme out here that is the apple extract we are adding is 150.

So, apple enzyme is actually 150 and our subscript catechol is 30. The basically we have to pipette out 150 plus 30 that is 180 microlitre of buffer from here for that we have taken this micro pipette and we have adjusted the volume to up to 180 microlitre we are taking out carefully from here this buffer 180 microlitre buffer we are taking out, ok. In the beginning we will be adding 30 microlitre of our catechol solution. So, I have taken this micro pipette and a fresh tip, ok. So, this is the catechol solution; the catechol solution we have taken out is this one. So, we are pipetting out this catechol from here, 30 microlitre catechol and we are adding here in this solution and we are mixing it after addition.

Now, after that what we need to do is that you need to add the protein. Always use a fresh tip for the new solution or new enzyme extract. Now, the protein we are going to take is actually 150 microlitre. We have adjusted this micropipette to 150 microlitre. We are taking a new tip from this tip box and this is the protein solution as you have seen. Now, one important thing to remember is that once you take this protein solution add it immediately in this mixture and immediately start the experiment.

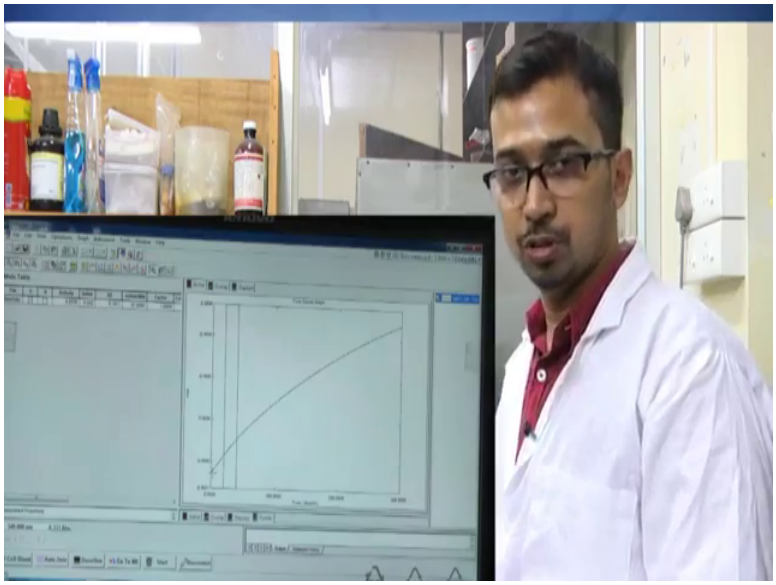
Please do not give any time delays for this experiment after or once you added the protein to it. So, now, we have taken this protein solution from here. We are opening this lid initially and here we are taking this cuvette putting this tip and will releasing the solution and up and down few times and immediately transferring it here closing this and start. It takes some time for the absorbance to show up in this screen. Here we can see with change in time and absorbance value is showing, but this is not actually the correct absorbance. We will get the value once the experiment is done with.

So, here we can see actually the weight is gradually increasing, the reaction is proceeding graphically. This appears to be somewhat parabolic where it gradually increases, but increase is not that steep rather smooth increase. What happens is that the reaction is actually taking place and this catechol is actually been oxidized in presence of

enzymes and (Refer Time: 16:03) the absorbance at 540 if changing which will be reflected from this curve with time the absorbance is changing.

So, almost we are done with 250 seconds and 50 seconds more for the reaction. Now, we can see here it is becoming more or less smooth out here. Initially there is a increase and it is becoming a bit smooth out here. Now, even increase in the absorbance value with time and will get a result after this experiment and 297 second 98 and 300 seconds. So, here are certain options or other clue which we can click, ok.

(Refer Slide Time: 17:33)



So, what we can see is that when we click this option out here, the main table actually, I would should some values and the value which here we should be actually bothered is that the activity given here or the milli absorbance per minute. So, these two values are actually the thing which you should be bothered because they are actually what they represent is that they represent a change the change in the absorbance with time, and the change is actually over this range, ok. This two are showing this 20 to 40, 20 second to 40 second over this range this change is shown. So, milli absorbance per minute it is been shown.

So, what you can do is that initially you need to save this one, ok. For saving you go to File, Save As. I am giving it sample sam 1 underscore catechol. So, this is the first sample for catechol the saving type is in case of a time course dot kin, kinetic file mode and again we can save this as a text file, it is a data printable whether you saved or not

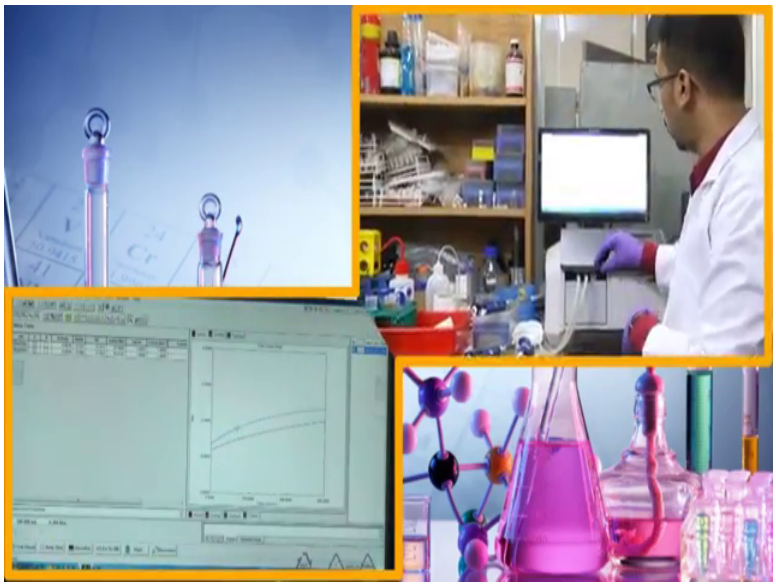
saved this one this is not important; however, what you need to do you need to record the time. So, what I will do is that I will record both the activity, and the milli absorbance per minute. So, the serial number is 1 and the activity I am getting here is 0.0576 and the milli absorbance per minute out here we are getting is 57.6092, ok. So, I am recording this one.

One important feature from this curve has we can see is that this is somewhat a parabolic and it is much similar to that of the curve in case of Michaelis-Menten equation all the both are not the same because in that case it was the velocity versus the substrate concentration out here it is absorbance versus time still it appears similar. However, here we can see the absorbance change in with time which we can say found this type of curved graph.

Now, let us shift to the second sample where will be using a higher concentration of CCMP. Now, we are going to use 2 milli molar of catechol. For that we require 60 micro litre of catechol and total volume is 600 microlitre and the buffer required is 390 microlitre and the enzyme extract the apple extract is actually 150 microlitre. Now, first let us use this phosphate buffer initially will be taking 390 microlitre of phosphate buffer. This is the phosphate buffer we are taking 390 microlitre. Now, one important thing is that of you have used this cuvette for previous sample you have clean it properly, so that you have to be sure that the apple enzyme is not sticking to this cuvette, ok.

So, we are removing the previously used tips and the next thing we will be pouring out here this our catechol, that is 60 microlitre. So, we are adjusting this to 60. We have taken a clean tip from here and this we are taking this catechol solution 60 microlitre of catechol solution and mixing properly out here and the next important thing is there we need to add 150 microlitre of protein. So, as I have discussed previously this is quite crucial and should be done in a rapid manner and, but be careful do not damage this cuvette or all the things while doing it in a hurried way. So, here we have taken again this apple extract and this is the cuvette here adding here thus one or two times I am mixing we are putting in the compartment, closing it and again start.

(Refer Slide Time: 22:50)



So, here we can once again we can change this one and the upper limit also. Here we can see this absorbance increases once again. However, the value of this absorbance is quite higher from the previous one since we can say the higher concentration catechol was used here, but let us not be to sure out here because when we are bothered with the activity part and the activity value and the milli absorbance per minute value.

So, the change in those values actually what determines the weight of the reaction. For the time being we can see that this curve is also similar to the previous one. The time out here is actually 300 seconds or 5 minutes. You can vary this time depending upon your convenience. Generally a longer time period actually gives a better result, and in all the cases equation study have shown that higher time period gives a move accurate result in case of enzyme kinetics reaction.

For the time being let us discuss about this main table out here. So, this one is the activity. So, what is the activity? If you have seen you know theory classes the activity of the enzyme. So, what is the activity of the enzyme? Enzyme activity refers how enzyme is working. Well, activity depends on many factors; activity depends on its substrate, activity depends on the type of enzyme, activity depends on the structure property of enzyme and many other factors out here. In this case actually the activity depends on the substrate because we are adding the substrate, but if we vary other parameters like temperature, pH shall the activity change. Well, you can decide because in a theory

classes we have seen how the enzyme stability changes on pH or other temperature and activity is no doubt related to the structural moiety of the protein. So, if there is any changes in the structure of the protein the activity will surely change.

Out here now, out here the parameters like temperature and pH are all same, now the pH we are using is 7.4 is pretty crucial out here and we will discuss a bit about pH after a few minutes. For the time being let us see how this curve goes. Again, it is attaining this almost flat shape out here, initially increasing and again the flat shape is 244, 245 and almost more or less around 50 seconds remaining.

So, it is almost 275 seconds are there and were we can see the increase in absorbance value is going, but however, the increases slowly drop it is rate of increase has dropped a bit after more or less 150 or 200 seconds as we can see here it is 295 and 5 more seconds. Well, we are done with this one and again we have to save this for that we have to go to save as sample 2. Here again data printable. Now, the important thing out here is we have to note this two the activity and the milli absorbance per minute out here. We can see the milli absorbance per minute is around 83 and whereas, the activity is 0.083, ok.

So, what happens is the both are actually same; the units are actually different out here, ok. So, the milli absorbance per unit rather the activity rater the reaction has we can see as increased when catechol has been added. So, again I am nothing down this values.

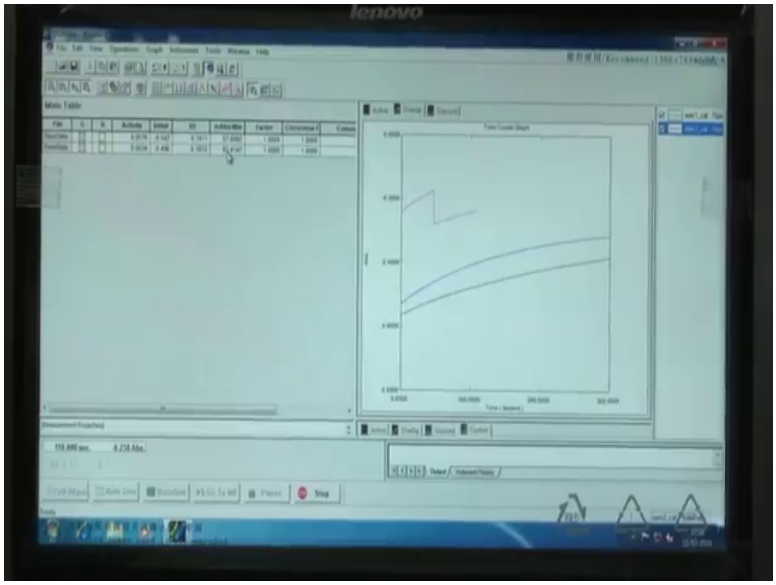
(Refer Slide Time: 28:59)

Now, once this experiment is done we have to take out this cuvette and if you take a closer look out here in this cuvette although I am not sure whether this is visible or not, but you can see this is the dark brown color or rather the yellow filled dark out here. So, it does not oxidize, ok. Now, we have to wash this cuvette properly if your are proceeding into the next analysis.

Now, we are done with 1 milli molar and 2 milli molar of catechol concentration; catechol is used as substrate. Now, we will use 4 mill molar of catechol; for that we will be taking the buffer as 330 microlitre of buffer. We have fed this pipette to 330 microlitre and now, we will take this buffer here we have taken 330 microlitre of buffer. After that we have calculated the volume of catechol required. The volume of catechol required for 4 milli molar is 120 microlitre.

So, we have adjusted this micro pipette to 120 and we are taking this fresh tip from this tip box and this is the catechol solution we are taking 120 micro litre of catechol and we are adding out here and mixing it properly in phosphate buffer. After that again the crucial step will be adding protein, it is 150 microlitre of protein. We are opening this lid, taking a new tip and we are adding this protein one – two times and immediately transferring it through a chamber closing this lid and start.

(Refer Slide Time: 32:05)



Again, we have to adjust this one. Here we can see it is going upwards like this way. So, from here what we can say is that this concentration is changing the concentration of

catechol in the mixture is changing and more of more of catechol oxidizes is being formed. However, here we can see a small change in the volume or small change in the absorbance value; a certain fall. Actually these were some errors which generally happen while we are doing a particular experiment, but one thing we have to take in mind is that the milli absorbance per minute value or the activity value is constantly changing either increasing or decreasing with concentration of catechol.

Generally, why such a fault takes place at moments? What happens is that the solution sometimes the change or the substrate which is undergoing a change is formed at a particular place. Suppose, in a cuvette and there is a solution, it is been formed at a particular stage in a middle stage, ok. Just assume, just assume it is been formed at particular stage, and it is not actually been homogenous. What happens is that when the light falls on that particular sample, what it sees is that it sees the concentration of new product at that particular region and that ever level the concentration at that region is much higher is compared to others. And as a result the absorbance value is reported as much higher.

But, however, when there is actually a devolution of compound and the solution become homogenous in that case. So, what happens is that the absorbance value again drops; because the concentration at that part again decreases it becomes homogenous. However, the absorbance value drops the overall absorbance value and however, we can say sharp drop out here and again sometimes bubbles are formed in the medium which might cause this change. So, these are manual errors which are expected to happen once you start doing an experiment.

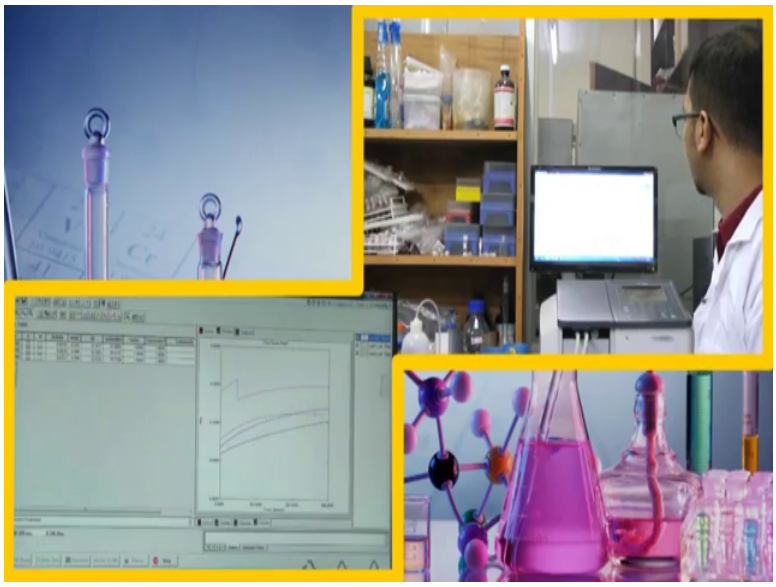
So, it is recommended often to use proper methods and proper precautions while doing this experiment. At times such jump in the absorbance value might lead to an error in the result. In such cases we have to repeat it again in the same procedures out here and in the other cases what you can do, you can take a large number of samples like you have what we have to do we have to actually calculate the milli absorbance per minute that is the velocity, ok.

Why it is velocity we are coming later on. So, milli absorbance per minute you have to calculate and you have to plot it in the y-axis and the x-axis we have to plot the substance concentration. So, at least you need 5 to 6 points. So, you can actually increase

the number of data of points to 8 or 9, why because in some cases the data point for other like for the second part or third part might be filled with errors or something like it you can actually omit those. So, here we can see it is becoming more or less smooth and 15 seconds remaining.

Now, what we have seen out here is that the value which comes is actually lower than the previous one, the milli absorbance per minute, it is around 75 whereas the previous one is around 83. So, some changes or problem might have occurred in that region as a result we are doing repeating this one again, we have taken the same amount of phosphate buffer and catechol and we are adding now 150 microlitre of protein solution. Taken this protein, now we are adding out here. As soon as we have added we are putting it here, close the lid and start.

(Refer Slide Time: 37:07)



Now, here we can see this is again increasing. Unlike the previous one this increment has started from this range actually. So, previous one started from here; now, it started a bit lower than that. Sometimes it happen it happen like it first initially increases and the rate again gets lower, and however, again there is an increase. So, actually until unless we plot the v max graph, we cannot actually estimate or cannot actually tell how the reaction is proceeding.

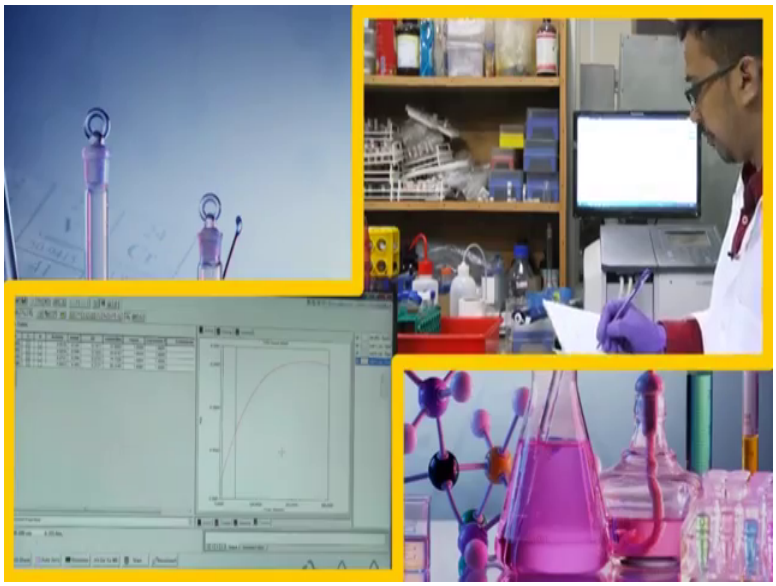
However, the steepness for this curve seems to be a bit lower than that of the previous one. So, let us see how it proceeds. See, sometimes what are the chances of error in this

reaction in this procedure? See, while we are doing this experiment errors might occur because the time range for which you are giving this mixing the sample in the cuvette may not be same in all the three cases. Number 2, the enzyme you are what you are using out here this enzyme, ok. So, it is been kept for a long time while you are doing this reaction.

Generally the proteins; the protein which you use should be kept in a very cold condition, ok. So, the enzyme there might be some changes in the enzyme or rather in those concentration of this entire volume. So, such changes are subjected to giving a defined value in case of our milli absorbance per unit or activity. Here again we can see it increases, but however, it is coming close to this one. Now, it is becoming almost smooth, here we can see fixed in this one a bit we can see this becoming smooth, bit a visualization is obtained from here. This shows that the reaction might have started a bit earlier before we have placed the start. There were certain changes, there are certain errors which might occur. So, the reaction must be repeated at least two to three times in cases. So, that in order to omit the result which come from errors, it is already 260 seconds and 40 more or 40 seconds to go out here.

This looks more or less like Michaelis-Menten curve out here. The absorbance value has become more or less static; rather it is changing very less. It is interesting this plot is quite interesting. Here we can see this one, it first increased and again it is becoming linear. Actually, this is the ideal plot; let us see how the value comes.

(Refer Slide Time: 42:07)

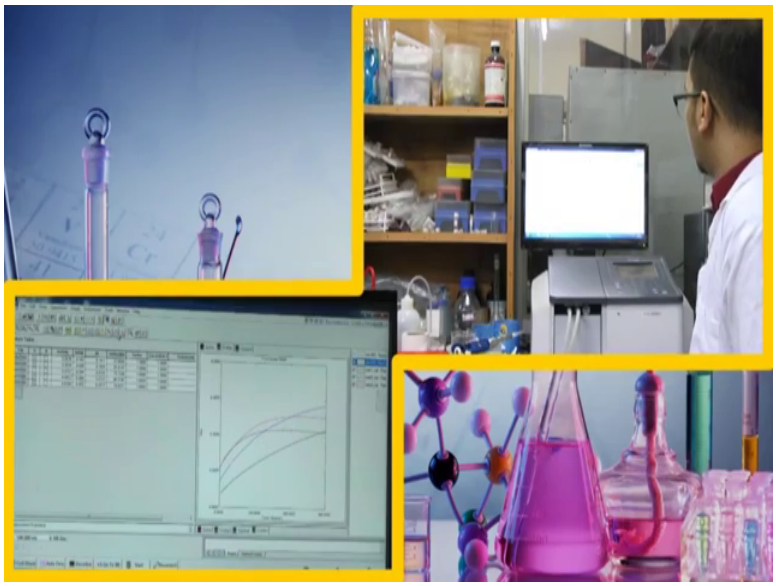


Well, the value comes around 1, however, if we change a bit. Generally, we can change this part this range, and we can see how this value actually changes the absorbance value. So, we can set a region where it is very steep. This region is quite steep, ok. So, this is basically around it is less than 20, but it is in a possible regions. So, it comes around 85. So, here we can see the constant the milli absorbance value or the activity as actually increased. So, we have to again save this one as sample 3, data text file and we are again noting down the activity is 0.085 whereas, the milli absorbance per unit is 85.31, previously it was 83 now, it is 85.31.

Now, what we can say is that in the previous one this it showed a steep increase initially that is why we get a much higher value in this case. So, let us move to the next concentration. Now, we are using 6 mill molar of catechol. For that we have taken 180 microlitre of catechol, ok. We have initially filled this cuvette with buffer, we have taken around 270 microlitre of buffer and 180 microlitre of catechol. So, we are taking again clean tip, these catechol solution taken 180 microlitre of catechol, you are mixing this one properly. Now, the prime important thing, now before adding this protein I would like to clean the sides of this cuvette because after adding protein it is better not to do all those stuff, ok.

So, now we have to open this lid, if the apple extract taking this one, taking close to this chamber adding up down, putting it inside and started it.

(Refer Slide Time: 45:13)



Here we can see it is almost similar, will let us move that graph out here, ok. So, here we can see this line if you cannot actually visualize properly out here, this is the orange colored line and over this orange line here we can see another line is coming out here. This is basically here, we can see this line is going from here. This is for our present sample, here we can see properly. So, easily out here it is going upwards. Interestingly, what we can see is that it has initially increased a bit if we compare this one minus 0.6 here it have initially increased a bit, but again it has decreasing.

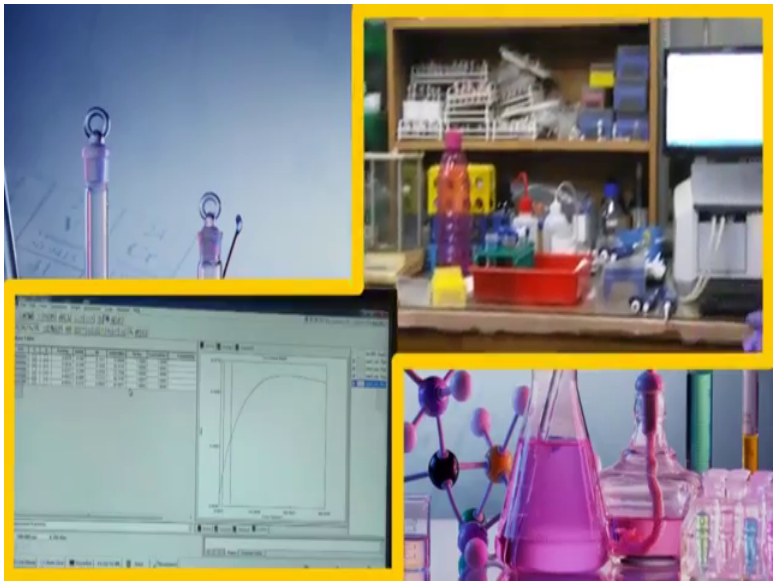
Well, what happens at higher concentration is that, at higher concentration this part is a flat part is basically saturated part. So, saturation is happening in the enzyme. What does that means? That actually means that enzyme or rather this one this catechol oxidize enzyme, the active side of the enzyme is getting occupied by this substrate. Now, as the substrate concentration is increasing all the proteins are getting all the active enzymes the active side of the enzymes are getting occupied. So, initially it is showing an activity, but however, since it is being saturated all the active sides are been filled up by or rather occupied, so it is getting saturation at a early stage clear. So, if we can compare the saturation from this two. We can see for the sample with around the blue line as we can see the blue line.

Here we can see the saturation is not that prominent out here it is getting saturated after 3 minutes, something or rather 300 seconds or something like that. what how a saturation

is not coming out here. On the contrary, we can see as we have increased the concentration of this substrate that is from 2 milli molar to 4 milli molar, this one is getting saturated out here and from here we can see it is getting saturated almost around if we could have drawn this line out here you can actually imagine it is getting saturated around 100 seconds. This one is the time, this is the second; around 100 seconds it has become saturated or over then that or more than or bit more than 100 second, that is the orange line.

However, talking about this deep brown line which is been operative currently for this sample for 6 milli molar we can see saturation has been started before 100 seconds, so, it is a interesting thing. Almost 235 seconds have gone and it is hardly moving here we can see the absorbance value is becoming static, slightly moving or it is more or less static. And, we expect the velocity change should not be much bit significant, we do not expect it to be a significant change out here because the upper one and the lower one more or less similar. So, it is 280 seconds and more than 280 around 15 seconds remaining, 2 seconds, 1 second.

(Refer Slide Time: 50:16)



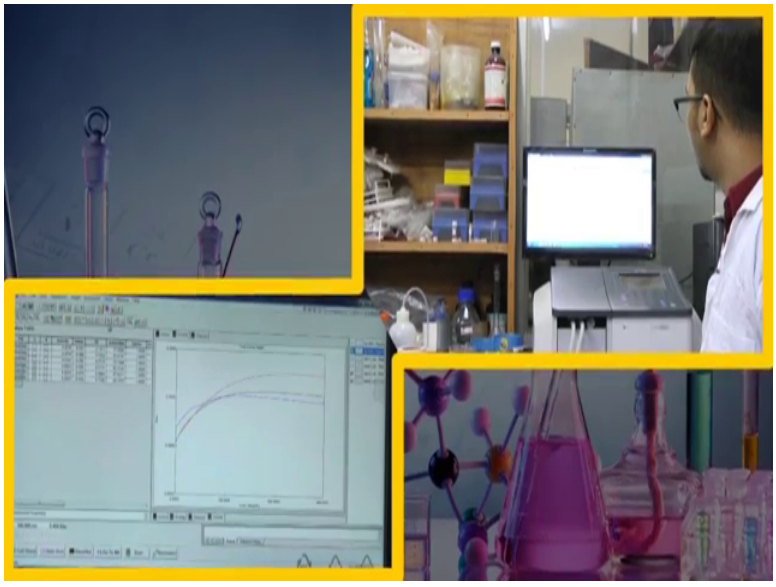
So, now it is over. So, we can actually move this one and see in which range we get an optimum value, ok. Out here we can see, we are getting an absorbance milli absorbance per unit value of around 87. First let us save this one as sample 4 in a text file, sample 4. So, here we can see it is around 87. So, I am noting this down for 6 milli molar for

sample 4 it is 0.087 activity and the milli absorbance per minute is 87.43. The previous one was 85 and this is 87.

Now, we will proceed with 8; will proceed with 8 milli molar of catechol solution. Now we will be dealing with catechol of concentration 8 milli molar, for that we are taking 240 microlitre of catechol, ok. Now, for 240 we are taking actually 120 microlitre 2 times because this is 200 microlitre maximum volume, 200 microlitre micro pipette. So, we are taking catechol 2 times, one we are adding here, we are mixing it. Well, I am using a fresh tip you might not use it because the catechol out here is phosphate buffer also, but here also in phosphate buffer.

However, sometimes what happens actually protein might there be contaminated, so, better to use a fresh tip. Now, I am using here the next thing I would be adding is protein that is 150 microlitre protein, this one is a protein solution. Well, I have taken this protein solution out here, before adding it I am cleaning the sides of the cuvette, now I am this adding this protein solution gradually out here and then up and down two times and then start.

(Refer Slide Time: 53:06)



While here we can see this one is increasing this value should be minus 0.6, yeah, it is better. So, here it is increasing and let us see what happens. Well, it is increased a bit. I am talking about this part the one in violet, here this one, ok. Now, I think again we are

getting this interesting result. It has increased a bit, now it is becoming a bit smoother yeah for it is gradually increasing.

However, more or less what I can predict or what we can see from here is that it has become more or less flat; more or less flat, and now it is intersecting with the previous one. If we remove others from here we can see that it has become almost in a plateau type of shape, and interesting thing what we have seen in the earlier case this saturation is becoming less than 10, 100 seconds this the 100 seconds. However, in here the saturation was coming at a value which is lower than this one because here we can see at this point for the brown one. Let us increase resolution 0.4 ok. 0.4 and 0.3, ok fine.

So, here we can see here at this point, at the point cross this junction is occurring at this point the saturation has started forming for the previous one that is for this deep brown color. But, however, in this case the blue line or the violet line we can see the saturation has started somewhere around here which is around 50 or something, 50 seconds or something. So, this shows that the saturation has been formed at a early stage when concentration is increased.

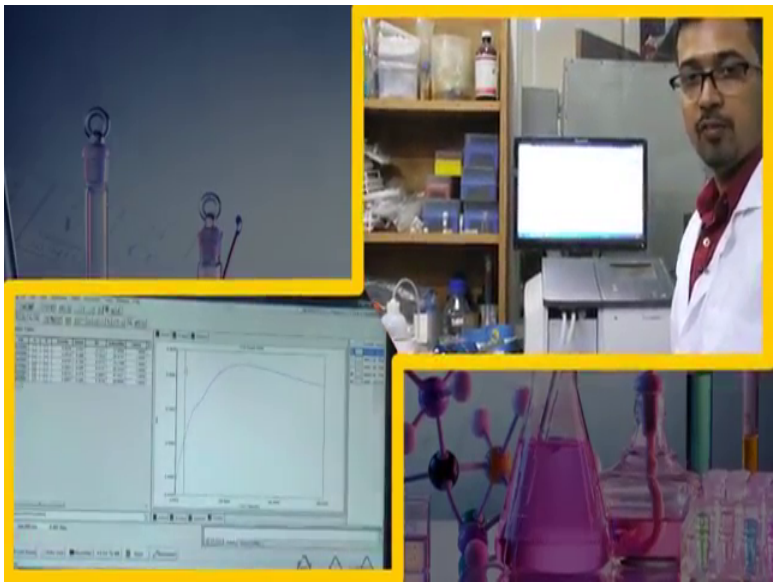
So, we are predicting for like 4 milli molar or 6 milli molar the saturation has coming and again for 4 milli molar let us see what what it is. Here we can see the saturation for 4 milli molar something where this point, for 6 milli molar it comes around this point, 4 milli molar it is over 100 seconds basically in a contractive way. Over 100 second for 6 milli molar, it is just less than 100 seconds, ok.

However, for this 8 milli molar what we are getting it is around 50 seconds. Here we can clearly see this one. So, this is quite sure that what actually happens is that protein at the in the medium where the enzymes are there which can actually clever catalytically oxidize this catechol substrate ok. Now, what happens is that if the catechol substrate is increasing, if the catechol substrate is increasing and all the active sides are getting blocked as I have discussed or rather all the active side not blocked all the active sides are getting saturated and as a result saturation is coming at an early stage. So, as the concentration is increasing more and more enzymes are getting saturated. So, the number of free enzymes are decreasing as a result the saturation is coming at an early stage.

Now, what we can do is that we have started with 1 milli molar, so, we can actually shift to 0.5 milli molar, ok. So, we can see how this comes to 0.5 milli molar. So, it is 268 it is

coming 270. Now, regarding pH what I was talking is that in case of pH when pH changes, ok, we were doing it around pH 7.4. So, pH changes pH increases or decreases it might affect the structure of the protein and there by enzymatic activity. Well it is a 300 seconds is there.

(Refer Slide Time: 58:08)



And, we can actually regulate this. We can actually alter or rather select a particular concentration a particular range where we have to actually select the activity. Well, the activity at this range is coming around more or less around 89, ok. So, I am noting this down, for 8 milli molar 0.0897 and 89.668. Now, I am doing this with 0.5 milli molar of catechol. Now, although we have started with 1 milli molar of catechol; however, out here we are taking 0.5 milli molar catechol the concentration of catechol here is 0.5 milli molar and for that we are taking 15 microlitre of catechol sample.

Now, this is a 20 microlitre pipette and we have adjusted it to 15 microlitre. Now, I am taking this one, adding this 15 microlitre catechol and after that I will be taking this protein once again. I am cleaning the side of the cuvette first, then keeping this lid open and then ready. Now I am adding this protein carefully, added this one and now start.

(Refer Slide Time: 60:24)

0.6 and it is minus 0.2. We have completed the kinetic analysis that is the enzyme kinetic study of catechol oxidize or rather the apple extract along with catechol, ok. So, now, what we need to do is that we need to add one inhibitor and see how our milli absorbance per minute value or activity value changes. As inhibitor we know that inhibitor actually blocks the active side of the enzyme and the substrate whereas, could not actually attach with the active side. Basically, depending upon the comparative or non comparative inhibitor the substrate attaches accordingly or rather the inhibitor attaches accordingly, ok.

So, if inhibitor attaches active side with increasing concentration of substrate in case of comparative inhibitor we can get the v max as we have seen from the theory classes. For that, what we can do is actually? We can actually change our experimental procedure, ok. So, how to change experimental procedure? One thing we can do is that we can just change the way in which we are adding the individual constituents. For example, initial we are adding phosphate buffer, then catechol and after that protein. Now, in doing so, what we are actually doing is that the protein is acting on catechol.

Now, we can proceed with this experiment in two ways; number 1, you are adding phosphate buffer, then catechol and then we are adding the enzyme rather the apple extract. And number 2 is that we are taking the phosphate buffer, then adding the enzyme and then adding the inhibitor. What is the difference in two cases? Basically what

happens is that when you are keeping enzyme and inhibitor or rather yes, enzyme the apple extract and inhibitor that is we are using here para hydroxybenzoic acid in the cuvette. What actually happening is the inhibitor is attaching to the active side of the enzyme.

And, now when we are adding substrate out there, that if the substrate means our catechol; so, what is happening if that it cannot actually get into the active site at that moment; however, if you are increasing the substrate in might go interactive site if it is comparative, ok. If it is non-comparative or uncomparative some other phenomenon might take place.

On the other way what happens if we add this inhibitor and catechol both at the same time in the cuvette and then enzyme we are adding, there might be a competition whether which one will be added the enzyme the substrate or the inhibitor. So, there might be a competition, both process are equally accepted and all the experimental data which are carried out in this research activities both process have been tried out. There are sometimes failures, sometimes some positive feedback from all this procedure, now in this case what we are going to see we were going to take the normal procedure. What is normal procedure? We are taking the enzyme, we were adding the inhibitor to it and we are keeping it and after that we are adding the substrate.

See the result may come or may come in this way, but before you approach with any inhibitor you have to try two ways whether it comes in the first way when the second way it depends upon the plot. In our case we will go with the first way in which we are taking the cuvette, ok. In this cuvette we will be filling the phosphate buffer first and after phosphate buffer we will be adding enzyme and after enzyme we will be adding the inhibitor that is hydroxybenzoic acid and after that will be adding catechol,. So, let us proceed with the analysis ok.

The concentration of inhibitor we are keeping here is around 0.8 milli molar or it is around 1 milli molar something like that. So, it depends upon the concentration of inhibitor. Out of the inhibitor it is around 0.8 milli molar and for that we will be taking 50 microlitre of inhibitor, but before that we have to take the phosphate buffer. The phosphate buffer will be taking out here is actually 370 microlitre and the catechol concentration out here is 1 milli molar like a previous experiment.

Now, I have washed this cuvette properly and I am taking the phosphate buffer, it is 370 microlitre then I will be adding the enzyme it is 150 microlitre of this enzyme I will be adding here. Say, until and unless you add this substrate catechol the reaction would not start theoretically. Now, I will be adding 50 microlitre of inhibitor that is para hydroxybenzoic acid or PHBA inhibitor and I am adding 50 microlitre of it. Two things you can do after adding, you can keep it for incubation for 1 to 2 minutes or you can directly add catechol to it. Whatever you do fix this time gap same for all the readings with all the with increment in catechol concentration.

Now, after that we are using the same micropipette with the different tip of course, for taking catechol we will be taking 30 microlitre of catechol. Now, out here we have to start as soon as we add catechol; so, opening this lid, taking catechol here mixing it and start.

(Refer Slide Time: 67:30)

Well, here we can see the absorbance value is increasing. The main thing we have to concentrate out here is the milli absorbance per minute and this is actually the velocity. Now, when we are plotting this experiment so, what we are doing? You are taking a velocity in the y-axis and substrate concentration the x-axis from plotting Michaelis-Menten equation from where you can get the v max value and the k m value? The k m value is better from line block, however v max value you can get from here and for that we will be doing is that we are in the velocity.

So, what is velocity? Velocity is the rate of a reaction as we know; and what is the rate of the reaction? It is actually rate is the change in concentration with time. So, rate means with time. So, rate of reaction means change in concentration with time. However, here we are not measuring the concentration, we are measuring the absorbance. So, why change in absorbance is referred to as rate? Because as we know, from Lamberts-Beer law that absorbance depends on concentration, ok. a equal to epsilon into c into l. So, that is absorbance is directly rational to the concentration.

So, the change in absorbance basically indicates the change in concentration. So, the absorbance for taking the rate we can take c 2 minus c 1 that is the concentration for at time 2 minus oscillation at time 1 divide by t 2 by t 1. Instead of c 1 and c 2 here we can take a 2 and a 1 that is a 2 is actually the absorbance at time 2 and a 1 is the absorbance at time one and this is actually what is giving milli absorbance per minute at this time is actually the velocity that is the change in absorbance with time.

Now, apart from that what you can do for this is that the value is been provided by the software. But, if you want to wish to get a much better results what you can do is that you can take a particular time range from here and you can actually subtract the absorbance manually. From the text file you can get the absorbance for a particular time, you can actually subtract it and for the change in absorbance with change in time you can also do it manually. What previously; in previous instruments we which did not have such facilities they used to do it in this way, where they manually used to plot it and from there they used to take those values and calculate the velocity.

Now, here we can see this is increasing with time which indicates that there is a change in the concentration of the component which is been formed, ok. So, actually here it is increasing this means the component is been formed that is the oxidation is been promoted more and more and the enzyme is acting on it. So, catechol is been oxidized to benzoic acid or rather para benzoquinone not benzoic acid it is para benzoquinone or basically for catechol it is just normal benzoquinone ortho benzoquinone for catechol, ok.

So, in case of benzoquinone what happens is that it has a, it absorbs it have wavelength ok, around 540 and this the more of more benzoquinone is being formed in the medium the more absorbance increases and from here we can get this increase in absorbance

value. So, almost 250 seconds have passed and see here we cannot see that any significant saturation in this case. However, the value is not that much increasing, saturation might be coming out here because change in absorbance is not that much prominent in this case. Now, we have to see what the activity data shows. Well, interestingly we can see here the milli absorbance per minute is reported out here is 34, which was in case without inhibitor it was 57, ok.

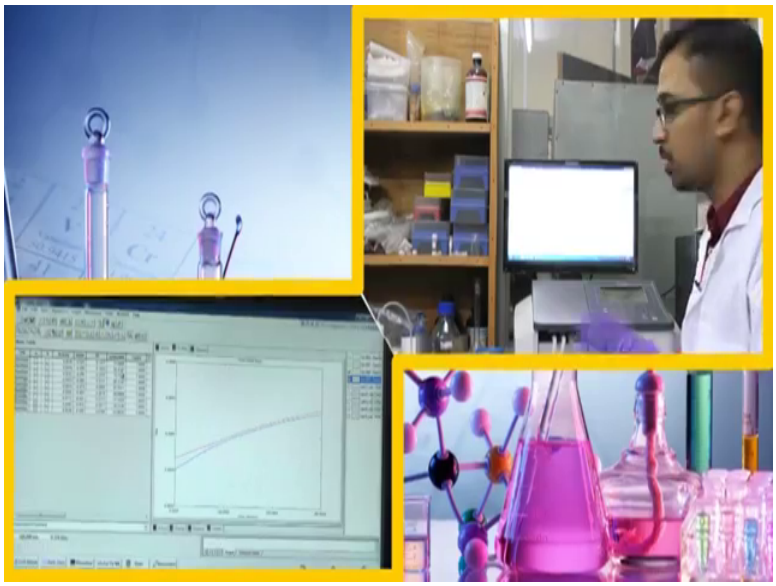
So, what we can see is that in presence of inhibitor, the activity or the rate has decreased there you can see the activity this 0.034 to any other activity is 0.05, ok; of course, 0.05 for the enzyme was much greater without inhibitor (Refer Time: 73:01) inhibitor decreased to the 0.03. So, again we are noting down this value in presence of inhibitor with pH para hydroxybenzoic acid for serial number 1, we are getting an activity of around 0.0342 and milli absorbance per minute which is 34.21.

Now, we will keep the concentration of inhibitor same out here and will vary the concentration of substrate because when we are plotting we are plotting the change in the concentration of substrate. Sometimes people might do it by mistake instead of changing the concentration of substrate this changing the concentration of inhibitor. But never do that always we are bothered with the rate at particular concentration of the inhibitor. So, out here the concentration is 0.83 milli molar and we are changing the concentration of catechol now.

Next we are taking 2 milli molar of catechol, for that we will be taking 340 microlitre of this buffer phosphate buffer 340 microlitre and taking in this cuvette and now the amount of inhibitor it is pH b is 50 microlitre. So, it should be same, but before that we have to add actually the protein and is 150 microlitre. We have taken 150 out here and this is the protein and adding 150 microlitre. For that we are adding this inhibitor, 50 microlitre inhibitor and kindly mix it carefully; and after that what we are doing? We are adding catechol and the volume of catechol is 60 microlitre.

So, before adding catechol once again what we should do? Should keep this lid open, wash the sides of this cuvette, add to the addition and take catechol and bring this close to this sample compartment add this one, I know 0.6..

(Refer Slide Time: 76:06)



Well, here we can see just started increasing, but the value is a bit lower than out here, but as we know as we have said earlier that in the absorbance might start increasing from a lower value higher value depending on when you are keeping the sample because at all the time at the same time we are not keeping it here. So, the thing we should be concerned is the milli absorbance per minute value.

So, let us see what it comes. Now, we can see here this one is increasing a bit and it is coming close to the previous one, the previous line. Generally, what happens is that when inhibitor occupy the active side of the enzyme it either allows the substrate the bind with it may not allow the substrate to bind with it or sometimes it may not happen it changes the conformation of the protein, ok. So, depending upon then if the conformation of protein changes. So, what happens is basically the substrate might not bind with it anymore and such type of inhibitors are generally referred to as suicide inhibitor, ok.

So, why because they bind with the protein in such a way, these are not the suicide inhibitor they form a covalent linkage with the protein, and they bind it, they bind they actually block the protein activity. So, sometimes what happens is that neuro concentration some neuro transmitter or these are enzymes or there some enzymes during the neuro transmitter and all those. So, if any types of poisonous gas comes within or either attaches to those enzymes what happens is that it blocks those enzyme and such a as a result our neuron fails to function or whether body or activity fails to function

which leads to fatal out comes in our body and sometimes people might not even continue living in such cases.

So, what happen with inhibitors or sometimes good, sometimes bad; inhibitors are good in cases when they are used as drugs, ok. So, what actually these drugs does something which blocks the malfunction of an enzyme, suppose an enzyme is acting in such a way in which it should not act or rather it showing some excess of it is function like an enzyme should have an optimal activity; if excess of function is showing then it is also harmful, if it is lower activity is showing it is also harmful. Suppose, an enzyme shows an activity which is very higher which is not desired actually, ok; in such cases what happens is that you such need something we should block those enzymes and that cases inhibitors are promoted in the form of drugs, ok.

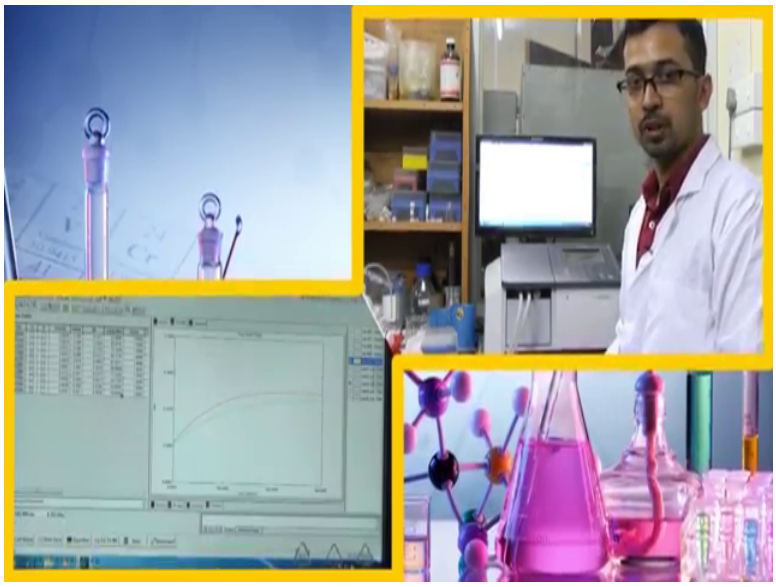
So, in our body I also discussed a bit of this in the previously. In our body what happens if any diseases is there it might be sometimes due to malfunction of some enzyme. So, drugs are promoted which actually blocks those enzyme. In doing so what happens is that drugs might actually interfere other enzymes, ok. So, as a result other enzymes which are not actually targeted the drugs are not targeted in that enzymes, they might not function properly. As a result what happens when we take too much of antibiotics or drugs it generally hampers our body function regular function, ok.

So, it is advised not to take so much drugs, they are ultimately the inhibitor of enzyme and all the inhibitors are not that much specific in cases. So, here it goes around 300 seconds. Well, interestingly again we can see the activity it is showing here is 53 as I said here it should be it is higher, but; however, it is going lower than this red line and is not a big issue we are concerned with the milli absorbance per minute activity it is showing 53. However, for the same concentration of catechol without inhibitor it was actually 83, ok. So, we are noting down the milli absorbance value 0.053 and it is 53.834 milli absorbance for value.

Now, we will move with 4 milli molar of our catechol, ok. Now, we are taking this catechol of 6 milli molar concentration rather sorry 4 milli molar concentration, for that we are taking 280 microlitre of buffer and then protein we are adding 150 microlitre rather the enzyme. Now, we will be adding 50 microlitre of our inhibitor, that is para hydroxybenzoic acid, we have mixed it properly and the catechol we require out here is

actually 120 microlitre. So, we are fixing it to 120 and before adding catechol I just opened this one, taken this catechol 120 I am adding here one-two times, done, close this lid and start.

(Refer Slide Time: 83:37)



Here we can see a green line of it, green line is appearing rather as in this way is green line is appearing which is for the absorbance change in absorbance with time. Basically, we have to go on like this for a particular concentration of substrate. We have to give add a particular concentration of the inhibitor. Now, it is better not to take the substrate concentration which you have not used without inhibitor part because in the first part. Suppose, I have taken 1 milli molar, 2 milli molar, 4 milli molar catechol and for inhibitor when we are taking with inhibitor; we are taking 1 milli molar, 3 milli molar, 6 milli molar or 5 milli molar catechol which was not there in the first set.

So, what happens in this case is that you cannot actually properly correlate, well you can get a proper experimental data also, but you can actually correlate like the way we were doing out here. It is better to take those concentration of the substrate in presence of inhibitor which we have taken earlier without inhibitor. So, here we can see there is a small fluctuations are out here. Actually what happens is that this experiment is good why because we were actually seeing something which we regularly observe, but we do not or rather cannot quantify, because we never ask any one that you are seeing apple is getting oxidized and turn brownish.

Tell me the rate at which it is getting oxidized? Well, people would not answer such questions also and because they do not have this UV setup. Well, you can actually get answer by yourself by doing this simple experiment where you do not have to buy expensive enzymes or expensive protein or this catechol is also very easily available and you can actually get this apple extract in a easy way and looks good basically.

However, some drawbacks of this experiment what you can actually think of is that in the in a particular fruit, there is not only one type of protein or rather one type of enzyme in the apple extract; there may be multiple enzymes at there in a fruit ok. So, the action of different enzymes on the substrate is not at all evaluated in this case. What we are getting the result is actual the combination of different enzymes on catechol. It is not the combination of only catechol oxidize on this enzyme, ok; number 1.

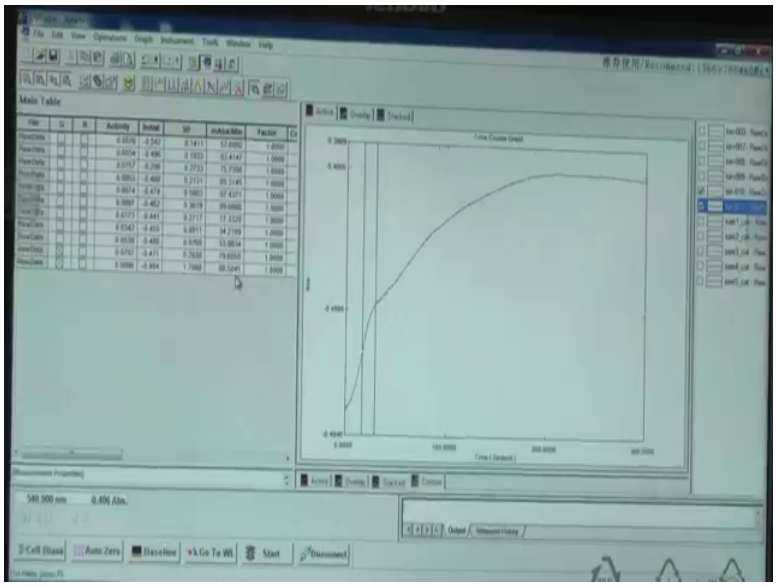
And, number 2 is that we do not know the concentration of catechol oxidize out here because we have taken particular amount of apple from this case. So, we need to actually quantify the amount of protein we are adding, when you will be doing any enzyme kinetics with whatever enzymes (Refer Time: 86:53) or (Refer Time: 86:54) you need to know the concentration of the enzyme, ok. So, concentration of enzyme is very much required out here.

These are few of the drawbacks and there is another drawback out here is that, when you are adding this or rather I am not telling about this experiment where there entire kinetics steps of that when you are adding all this we have to complete take a monitor of the time you are adding. So, manual error might occur, it is expected that manual error might take place in this way. So, in order to avoid those manual error some other techniques should be introduced in other in order to study a kinetics.

So, here we can see saturation has started occurring. Here it is coming, this one for sample 3, this orange color was for sample 3. I did not actually save the others so, you cannot actually see here. Now, it is coming here it is see it is much lower, the steepness is much lower than that of the sample where catechol was present without any inhibitor, this one was with without inhibitor and this one was with inhibitor. Well, out here we can see the rate is increased to 79 or rather activity 0.079 and the milli absorbance per minute is 79.

So, the milli absorbance per value is again increased. However, in the previous without inhibitor the value was 85 as I have noted previously here the value is 0.079 and it is 79.65. The activity is gradually increasing with increase in concentration of catechol; however, this is not the same as that where inhibitor was absent, that rate was much higher. Now, let us proceed and see what happens for 6 milli molar of catechol concentration.

(Refer Slide Time: 89:27)



In this case what we have seen; in this case what we have seen is that it goes to the value of around 80.58 the milli absorbance per minute value. So, in the previously it was 87 as we have seen earlier it has 87, now 0.086 and it is 80.58. Actually in this way what we can do is that we can actually go on increasing the concentration of the substrate and from there what we can say is that we can get the V value.

Now, these milli absorbance per minute value are the V that is the velocity ok. So, we need to is that we need to take those velocity and plot it in the y-axis for a particular concentration substrate. We will get something like this, initially as we have seen a in this data is started from 57 and it go high to 83 or something like that then 85 and 87 and almost it is becoming linear like the Michaelis-Menten.

Similarly, in this way it is started from 34 without inhibitor and then went to 53 from a data I am saying and then 79 and then 80 and now again it is decreasing. So, well, once we plot this graph at very high for very high concentration of substrate we can see

whether it reaches the v max or not from there we can calculate whether it is comparative or non comparative inhibitor.

Well, so, what we have seen in this experiment? We have studied how to carry out enzyme kinetics reaction. Initially we have prepared catechol solution, para hydroxybenzoic acid solution and phenylthiourea, but phenylthiourea we can use also and see how the kinetics changes. You can actually compare it to different rates and see whether for a particular concentration of catechol which gives high velocity for phenylthiourea or for para hydroxybenzoic acid. If the V value or the rate is higher for phenylthiourea; that means, para hydroxybenzoic acid is better inhibitor since it decreases the rate, in this way you can compare. So, it is always better to use two different types of inhibitor.

Secondly, we have extracted the juice on the apple by grinding it in a mortar pestle and we have used it we have actually filtered it using a cloth paper. Sometimes you can actually centrifuge it and we can use the filtrate from them and; however, we have seen it is almost a clear solution. So, we can easily use that filtrate and you have used it as a source of protein. Initially we have taken the phosphate buffer pH 7.4 and 10 milli molar; you can use 20 milli molar also and that phosphate buffer. What we have done is that from phosphate buffer we have taken, the phosphate buffer we have taken in a cuvette 10 milli molar of pH 7.4 phosphate buffer and to it we have added initially catechol and then protein and immediately given it for scanning.

Now, in case of inhibitor what we have done we have taken the buffer added protein first, then inhibitor and then we have taken it close to the instrument and added catechol at that moment. So, I am not this is the ideal way of doing the reaction, you can do it in other way you can add enzyme and like before adding enzyme you can add inhibitor and catechol you can see it yourself. Once you start doing the reactions by yourself you can apply whatever method you think will be best. For the time being I hope this was quite fruitful and how to carry out with enzyme kinetics the basic step you have learnt.

Thank you.